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Interaction between Two Putative Glycosyltransferases Is Required for Glycosylation of a Serine-Rich Streptococcal Adhesin▽

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Fap1, a serine-rich glycoprotein, is essential for fimbrial biogenesis and biofilm formation of *Streptococcus parasanguinis* (formerly *S. parasanguis*). Fap1-like proteins are conserved in many streptococci and staphylococci and have been implicated in bacterial virulence. Fap1 contains two serine-rich repeat regions that are modified by O-linked glycosylation. A seven-gene cluster has been identified, and this cluster is implicated in Fap1 biogenesis. In this study, we investigated the initial step of Fap1 glycosylation by using a recombinant Fap1 as a model. This recombinant molecule has the same monosaccharide composition profile as the native Fap1 protein. Glycosyl linkage analyses indicated that *N*-acetylglucosamine (GlcNAc) is among the first group of sugar residues transferred to the Fap1 peptide. Two putative glycosyltransferases, Gtf1 and Gtf2, were essential for the glycosylation of Fap1 with GlcNAc-containing oligosaccharide(s) in both *S. parasanguinis* as well as in the Fap1 glycosylation system in *Escherichia coli*. Yeast two-hybrid analysis as well as in vitro and in vivo glutathione *S*-transferase pull-down assays demonstrated the two putative glycosyltransferases interacted with each other. The interaction domain was mapped to an N-terminal region of Gtf1 that was required for the Fap1 glycosylation. The data in this study suggested that the formation of the Gtf1 and Gtf2 complex was required for the initiation of the Fap1 glycosylation and that the N-terminal region of Gtf1 was necessary.

The *Streptococcus sanguinis* (formerly *S. sanguis*) streptococci are predominant microbes in the oral cavity. They colonize saliva-coated teeth and other oral surfaces and play an important role in the development of dental plaque (2, 12, 18). Adhesion of *Streptococcus parasanguinis* (formerly *S. parasanguis*) FW213 in an in vitro tooth model is mediated by fimbriae (9, 11). *S. parasanguinis* fimbriae are composed of multiple subunits of a serine-rich glycoprotein, Fap1 (35–37). Fap1 belongs to a growing family of serine-rich proteins. This family of proteins include GspB and Hsa of *Streptococcus gordonii* M99 (1) and Challis (29), SraP of *Staphylococcus aureus* (25), SrpA of *Streptococcus sanguinis* (21), PsrP of *Streptococcus pneumoniae* (20), and Srr-2 of *Streptococcus agalactiae* (23). Hsa, SraP, PsrP, and Srr-2 have been implicated in bacterial pathogenesis (20, 23, 25, 30). Fap1 is a large molecule comprised of 2,572 amino acid residues. It possesses two serine-rich repeat regions (RI and RII) and an acidic amino acid-rich, nonrepeat region. The RI region contains 28 copies of the dipeptide motif, whereas the RII region consists of 1,000 copies of the same dipeptide motif. A cell wall anchor domain at the carboxyl terminus is essential for Fap1 surface presentation, fimbrial biogenesis, and bacterial adhesion (35). Fap1 is heavily glycosylated with *N*-acetylglucosamine (GlcNAc) and glucose residues within the serine-rich repeat regions (27). Glycosylation of Fap1 is important for the biofilm formation of *Streptococcus parasanguinis* (38).

A seven-gene cluster downstream of *fap1* has been identified and implicated in Fap1 glycosylation. One glycosyltransferase, Gtf1, is critical for Fap1 glycosylation (34), whereas two accessory secretion components, SecA2 and SecY2, are required for the biogenesis and secretion of mature Fap1 (3, 34). The serine-rich repeat regions are modified by O-linked glycan moieties (27). The compositions of the monosaccharides on Fap1 are known (27), but the order of transfer and the identity of the initial monosaccharide residue(s) transferred to Fap1 are not known. Other genes that are involved in the transfer and how they coordinate to initiate Fap1 glycosylation have not been investigated. O-linked glycan moieties are synthesized by glycosyltransferases in a stepwise manner (28). Eukaryotic glycosyltransferases and their related proteins interact with each other to form enzyme complexes in N-linked glycan biosynthetic pathways (6, 24). However, such interactions have not been identified in O-linked glycosylation systems (6). Both N- and O-linked glycosylation systems are found in a variety of bacterial species (28), including streptococci (8), and some bacterial and eukaryotic systems share a common transfer mechanism (33). However, whether bacteria have adopted protein-protein interaction schemes similar to eukaryotic cells to control protein glycosylation is unknown.

To address the above questions, we established an in vivo glycosylation model in *Escherichia coli* and studied the functional contribution of two putative glycosyltransferases, Gtf1 and Gtf2, to Fap1 glycosylation. Our experimental evidence suggested that GlcNAc was among the first group of sugar

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TABLE 1. Strains used in this study

Strain	Relevant characteristic(s)	Source or reference
S. parasanguinis		
FW213	Parent strain	5
VT1393	FW213 *fap1*::*aphA3*; Kan^r	37
VT1583	FW213 *gtf1*::*aphA3*; Kan^r	34
AL51	FW213 *gtf1*::*aphA3*/pVPT; Kan^r Erm^r	34
AL52	FW213 *gtf1*::*aphA3*/pVPT-*gtf2*; Kan^r Erm^r	34
AL50	FW213 *gtf1*::*aphA3*/pVPT-*gtf1*; Kan^r Erm^r	This study
AL63	FW213 *gtf2*::*aphA3*; Kan^r	This study
AL64	FW213 *gtf2*::*aphA3*/GFP-pVPT; Kan^r Erm^r	This study
AL65	FW213 *gtf2*::*aphA3*/GFP-pVPT-*gtf1*; Kan^r Erm^r	This study
AL66	FW213 *gtf2*::*aphA3*/GFP-pVPT-*gtf2*; Kan^r Erm^r	This study
AL74	FW213 *gtf1 gtf2*::*aphA3*; Kan^r	This study
AL75	FW213 *gtf1 gtf2*::*aphA3*/pVPT-*gtf1*; Kan^r Erm^r	This study
AL76	FW213 *gtf1 gtf2*::*aphA3*/pVPT-*gtf2*; Kan^r Erm^r	This study
AL77	FW213 *gtf1 gtf2*::*aphA3*/ pVPT-T7-*gtf1-2*-GFP; Kan^r Erm^r	This study
AL81	FW213 *fap1*::*aphA3*/pVA838-*fap1*ΔRII; Kan^r Erm^r	This study
Saccharomyces cerevisiae		
AH109	Two-hybrid screening host	Clontech
AL-Y2H1	Cotransformants of pAD-*gtf1* and pBD-*gtf2*	This study
AL-Y2H2	Cotransformants of pAD-*gtf1* and pBD	This study
AL-Y2H3	Cotransformants of pBD-*gtf1* and pAD	This study
AL-Y2H4	Cotransformants of pAD-*gtf2* and pBD-*gtf1*	This study
AL-Y2H5	Cotransformants of pAD-*gtf2* and pBD	This study
AL-Y2H6	Cotransformants of pBD-*gtf2* and pAD	This study
E. coli		
Top10	Cloning strain for cotransformants	Gibco BRL
AL400	Cotransformants of pAL81 and pGEX6p1	This study
AL401	Cotransformants of pAL81 and pAL200	This study
AL402	Cotransformants of pAL81 and pAL201	This study
AL403	Cotransformants of pAL81 and pAL202	This study
AL406	Cotransformants of pAL81 and pAL205	This study
AL407	Cotransformants of pAL81 and pAL206	This study
AL408	Cotransformants of pAL81 and pAL207	This study
AL409	Cotransformants of pAL81 and pAL208	This study
AL410	Cotransformants of pAL81 and pAL209	This study
AL411	Cotransformants of pAL81 and pAL300	This study

residues transferred to the Fap1 polypeptide, that Gtf1 interacted with Gtf2, and that this interaction was required for the transfer.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial and yeast (*Saccharomyces cerevisiae*) strains and plasmids used in this study are listed in Tables 1 and 2. *S. parasanguinis* parent strain FW213 and its derivatives were cultured using Todd-Hewitt broth (THB) with the appropriate antibiotics as described previously (38).

DNA manipulation and sequence analysis. Standard methods (22) were used for routine DNA manipulation. DNA-modifying enzymes were purchased from Promega (Madison, WI). Plasmid DNA was isolated using Qiaprep Spin Miniprep kit (Qiagen, Inc., Santa Clarita, CA). Genomic DNA from *S. parasanguinis* strains was isolated using the PureGene genomic DNA purification kit (Gentra Systems, Minneapolis, MN). The PCR primers used are listed in Table 3. Nucleotide sequences of amplified PCR products were determined by the dideoxy chain termination method with an ABI 1372A DNA sequencer (26). Homology comparison was carried out using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw/).

Construction of *gtf2* single mutant and *gtf1 gtf2* double mutant in *S. parasanguinis*. The *gtf2* gene with 500-bp flanking DNA sequence was PCR amplified from genomic DNA of *S. parasanguinis* using primers *gtf2*-5′ and *gtf2*-3′. The PCR fragment was cloned into pGEM-T Easy vector to generate pAL60. An inverse-PCR strategy (19) was used to introduce a PstI site into pAL60. In brief, primers with embedded PstI sites, *gtf2*-PstIF and *gtf2*-PstIR, were used to inverse PCR amplify a 4.20-kb fragment from pAL60. The amplified fragment was digested with PstI and self-ligated to generate pAL61, in which the *gtf2* gene was deleted. pAL61 was then digested with PstI and ligated with a nonpolar kanamycin resistance cassette, *aphA3* (a gift of Allen Honeyman) (14), to form pAL62, in which *gtf2* was interrupted by insertion of *aphA3*. The mutated *gtf2* allele was excised from pAL62 by EcoRI digestion and then ligated into *S. parasanguinis* suicide vector pSF143 (32) to create pAL63.

A plasmid used to generate the *gtf1 gtf2* double mutant was constructed in a similar manner. The *gtf1 gtf2* fragment with 600-bp flanking sequences was amplified using the primer pair Gtf-Spara-FF and Orf4D2-Spara-RR and then cloned into pGEM-T Easy to generate plasmid pAL70. The majority of *gtf1* and *gtf2* open reading frame (ORF) sequences were deleted from pAL70 by inverse-PCR amplification with primer pairs *gtf1-2*-HindIII-IVS-RR and *gtf1-2*-HindIII-IVS-FF. The amplified fragment was digested with HindIII and self-ligated to generate pAL71. pAL71 was then digested with HindIII and ligated with the nonpolar kanamycin resistance cassette *aphA3* to form pAL72, in which the *gtf1 gtf2* fragment was replaced by *aphA3*. The allele replaced by *aphA3* was excised from pAL72 by EcoRI digestion and then ligated into *S. parasanguinis* suicide vector pSF143 (32) to create pAL73. These plasmids, pAL63 and pAL73, were transformed into *S. parasanguinis* FW213 as described previously (10) to generate kanamycin-resistant and tetracycline-sensitive allelic replacement mutants. Insertional inactivation of *gtf2* or *gtf1* and *gtf2* by a double-crossover recombination event was confirmed by PCR and Southern blot hybridization. Confirmed *gtf2* and *gtf1 gtf2* mutants AL63 and AL74 were used for further studies.

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Source or reference
pGEM-TEasy	PCR cloning vector; Amp^r	Promega
pVA838	*E. coli-Streptococcus* shuttle vector; Tet^r Erm^r	17
GFP-pVPT	Modified pVA838 with maltose promoter; Tet^r	4
pAL60	*gtf2* with flanking regions in pGEM-T Easy; Amp^r	This study
pAL61	*gtf2* gene deleted from pAL60; Amp^r	This study
pAL62	*aphA3* gene inserted into pAL61; Amp^r Kan^r	This study
pAL63	*gtf2*::*aphA3* fragment cloned into pSF143; Kan^r	This study
pGEX6p1	Vector used to generate GST fusion proteins; Amp^r	Amersham
pAL70	*gtf1 gtf2* fragment with extra 600-bp up- and downstream sequences in pGEM-T Easy vector; Amp^r	This study
pAL71	Large portion of *gtf1 gtf2* fragment deleted from pAL70 by inverse PCR and self-ligated; Amp^r	This study
pAL72	*aphA3* gene inserted in pAL71; Amp^r	This study
pAL73	*gtf1 gtf2*::*aphA3* fragment cloned into pSF143; Kan^r Tet^r	This study
pVT1175	Full-length *fap1* in pHSG576; Kan^r	37
pAL80	RII deleted from pVT1175 with inverse PCR; Kan^r	This study
pAL81	HindIII fragment of pVA838 with an Erm^r cassette and streptococcal replicon ligated with pAL80; Erm^r	This study
pAL90	*gtf1* cloned in GFP-pVPT; Erm^r	This study
pAL91	*gtf2* cloned in GFP-pVPT; Erm^r	This study
pAL92	*gtf1 gtf2* fragment cloned in GFP-pVPT; Erm^r	This study
pAL200	*gtf1 gtf2* ORFs cloned in pGEX6p1; Amp^r	This study
pAL201	*gtf1* aa 21~485 deleted from pAL200; Amp^r	This study
pAL202	*gtf2* aa 15~417 deleted from pAL200; Amp^r	This study
pAL94	*gtf2* gene cloned into pET27b; Kan^r	This study
pAL205	T7 tag fused at C-terminal *gtf2* in pAL200; Amp^r	This study
pAL206	T7 tag fused at C-terminal *gtf2* in pAL201; Amp^r	This study
pAL207	Tn*5* transposon inserted in Gtf1 aa 31 in pAL205; Amp^r	This study
pAL208	Tn*5* transposon inserted in Gtf1 aa 65 in pAL205; Amp^r	This study
pAL209	Tn*5* transposon inserted in Gtf1 aa 159 in pAL205; Amp^r	This study
pAL300	Tn*5* transposon inserted in Gtf1 aa 416 in pAL205; Amp^r	This study

Complementation of *gtf1*, *gtf2*, and *gtf1 gtf2* mutants. The ORFs of *gtf1*, *gtf2*, and the *gtf1 gtf2* mutant were amplified from genomic DNA of *S. parasanguinis* with primer pairs SalI-*gtf1*-FF and BamHI-*gtf1*-RR, SalI-*gtf2*-FF and KpnI-*gtf1-2*-RR, and SalI-T7-*gtf1-2*-FF2 and KpnI-T7-*gtf1-2*-RR, respectively (Table 3). The PCR products were digested with KpnI and SalI or BamHI and SalI and then ligated into *E. coli-Streptococcus* shuttle vector GFP-pVPT (4). GFP-pVPT has a functional streptococcal promoter, *Pmal*. The desired plasmids were isolated from putative transformants and confirmed by restriction enzyme digestion and DNA sequencing. The control plasmid, GFP-pVPT, and the resulting plasmids, *gtf1*-pVPT, *gtf2*-GFP-pVPT, and T7-*gtf1-2*-GFP-pVPT, were transformed into the respective mutants. The ability of cloned *gtf1*, *gtf2*, and the *gtf1 gtf2* fragment to restore the mature Fap1 expression in their corresponding mutants was examined by BactELISA assays (34) using monoclonal antibody (MAb) F51 (27). The positive transformants were further confirmed by Western blot analyses with peptide-specific MAb E42 and glycan-specific MAb F51 (27).

Construction of Fap1ΔRII in *S. parasanguinis*. pVT1175 (37) contains the full-length *fap1* gene and an *E. coli* origin of replication. To construct Fap1ΔRII, An inverse-PCR strategy was used to delete the repeat region II from pVT1175 with *fap1*-1814KpnI and *fap1*-7080KpnI primers. The inverse-PCR product was digested with KpnI and self-ligated to generate plasmid pAL80. The resultant pAL80 was digested by HindIII and ligated with a HindIII fragment of pVA838 that contains a streptococcal origin of replication and an erythromycin resistance cassette to generate pAL81 (*fap1*ΔRII-pVA838-HindIII). pAL81 was transformed into the *fap1* mutant VT1393 to generate AL81.

Expression, purification, and characterization of Fap1ΔRII. Culture supernatants of AL81 were collected by centrifugation and precipitated with 60% $(NH_4)_2SO_4$. The precipitated proteins were subjected to buffer exchange against phosphate-buffered saline with Centricon YM-50 (Millipore), the concentrated samples were further purified with a MAb E42 immunoaffinity column which was packed with disuccinimidyl suberate (DSS)-cross-linked MAb E42 and protein G. The purification procedure was performed according to the instructions of the Seize X protein G immunoprecipitation kit (Pierce, Rockford, IL). The purity of the Fap1ΔRII was assessed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Coomassie brilliant blue staining.

Monosaccharide composition and glycosyl linkage analyses of Fap1ΔRII. The monosaccharide composition analysis procedure was carried out as previously described (15). In brief, methyl glycosides were prepared from 100 μg of the dried Fap1ΔII by methanolysis in 1 M HCl in methanol at 80°C for 18 h, followed by re-N-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were per-O-trimethylsilylated by treatment with Tri-Sil at 80°C for 20 min. Gas chromatography-mass spectrometry (GC-MS) analysis was performed on an HP5890 gas chromatograph interfaced to a 5970 MSD using a Supelco DB-1 fused-silica capillary column (30 m by 0.25-mm inside diameter).

Glycosyl linkage analysis of Fap1ΔII was performed with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS. Purified Fap1ΔRII was subjected to β-elimination with 1 M NaBH4 in 50 mM NaOH to release O-linked glycan(s). The released glycans were permethylated by the method of Ciukanu and Kerk (7) and extracted with methylene chloride-water to remove any impurities, the resulting permethylated samples were suspended in methanol and mixed with MALDI matrix 2,5-dihydroxyl benzoic acid. MALDI-TOF MS was performed with a Voyager mass spectrometer operated in the positive-ion mode. Profiling of constituent glycans was monitored.

Coexpression of Fap1ΔRII with two glycosyltransferases and their derivatives. The *gtf1 gtf2* fragment was amplified with BamHI- and XhoI-embedded primers listed in Table 3. The PCR product was digested and then ligated into pGEX6p1 to allow the in-frame fusion of glutathione *S*-transferase (GST) with *gtf1* to generate pAL200. Two negative control plasmids were also constructed. One lacks *gtf1*, and the other lacks *gtf2*. To delete the majority of *gtf1*'s ORF (amino acids [aa] 21 to ~485), we performed inverse PCR using pAL200 as a template and *gtf1*-KpnI-IVS-RR and *gtf1*-KpnI-IVS-FF as a primer pair. The inverse-PCR product was digested with KpnI and self-ligated to construct pAL201. A similar strategy was used to delete aa 15 to ~417 from *gtf2*'s ORF in pAL200 using the primer pair *gtf2*-KpnI-IVS-RR and *gtf2*-KpnI-IVS-FF to construct pAL202. Substrate plasmid pAL81 (chloramphenicol resistant) was cotransformed with pGEX6p1; two negative control plasmids, pAL201 and pAL202; or a positive control plasmid, pAL200 (ampicillin resistant). The chloramphenicol- and ampicillin-resistant cotransformants were further analyzed by the presence of two

TABLE 3. Primers used in this study

Primer	Sequence (5′→3′)[a]
gtf2-5′	CTTCTTGAGGAGCAATTCACAC
gtf2-3′	AGGACAAGATCAATAACGCA
gtf2-PstIF	GATCCTGCAGATCCAAAGATTCTTGTGTGAGC
gtf2-PstIR	CTGCAGGTTAGATTTACTGGAACAATGTG
gtf-Spara-FF	GCGAGGATAAGTCGGAATCTG
Orf4D2-Spara-RR	AGGACAAGATCAATAACGC
gtf1-2-HindIII-IVS-RR	TCGAATAAGCTTCCGATCAGAACTCGC
gtf1-2-HindIII-IVS-FF	TGACAAGCTTACATTATCTGATTG
SalI-*gtf1*-FF	TGACGTCGACATGACAATCTATAATATTAATTTAGGG
BamHI-T7-*gtf1*-RR	TGACGCGGATCCCTAATCATTTAACATCTCC
SalI-*gtf2*-FF	TGACGTCGACATGATAGTTTTTGAATGGC
SalI-T7-*gtf1-2*-FF2	GGACGTCGACATGGCTAGTATGACAGGAGGAGGTCAACAAATG GGAATGACAATC TATAATATTAATTTAGGG
KpnI-T7-*gtf1-2*-RR	TGACGCGGTACCATCTACATTACTAACCAATACCTGTTTATAATC
fap1-1814KpnI	GTTGAGGTACCGGTAGACTCTGATTTT
fap1-7080KpnI	GTTGAGGTACCGAAAGAACACTTCCG
BamHI-*gtf1*-FF	GATCGGATTCGAGGGAACAATGACAATC
XhoI-*gtf2*-RR	GATGCTCGAGGATTTTAACTACTAAATGGTC
gtf1-KpnI-IVS-RR	GACTGGTACCAGCATACTCAACACCGCTAC
gtf1-KpnI-IVS-FF	GATCGGTACCAAGCATGAAAAATTAGTTGAAAGATGG
gtf2-KpnI-IVS-RR	GACTGGTACCATCAAAGATTCTTGTGTGAGC
gtf2-KpnI-IVS-FF	GACTGGTACCTGGCGATCAGACTTATTTCCG
EcoRI-*gtf1*-FF	CGGAATTCATGACAATCTATAATATTAATTTAG
BamHI-*gtf1*-RR	CGGATCCATCATTTAACATCTCCTC
EcoRI-*gtf2*-FF	CGGAATTCATGATTAGGTTGTTTGAATGGC
BamHI-*gtf2*-RR	CGGATCCATCTACATTACTAACCAATAC
BglII-*gtf2*-FF	CAGGTTTAGATCTATCATC
pGEX6p1-*gtf1*-F(910)	AATCGGATCTGGAAGTTC
NotI/KAN-3 RP-2	TCCCGTTGAATATGGCTCATAAC
gf2-T7-XhoI-RR	TGACCTCGAGTCCCATTTGTTGACCTCCTGTCATACTAGCCATAT CTACATTACTAACCAA

[a] Restriction enzyme sites are underlined.

distinct plasmids. The strains harboring correct plasmids were chosen for the subsequent experiments.

Analysis of Fap1ΔRII glycosylation by lectin blotting assays. Culture supernatants of *S. parasanguinis* were prepared as described previously (34). Cell pellets from 0.5-ml exponential-phase-grown *E. coli* cultures were harvested and subjected to electrophoresis on a 4 to 12% gradient gel. Separated proteins were transferred to nitrocellulose membranes and incubated with blocking buffer, which contains 1× Tris-buffered saline (TBS), 0.1% Tween 20, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 2% polyvinyl alcohol (Sigma) for 1 h and then subjected to lectin blot analyses with horseradish peroxidase-conjugated succinyl wheat germ agglutinin (sWGA) or other conjugated lectins.

Yeast two-hybrid analysis. The procedure for yeast two-hybrid analysis was carried out according to the protocol of the MATCHMAKER system 3 (Clontech). DNA binding domain (BD) and activation domain (AD) fusion vectors, pGBKT7 (pBD) and pGADT7 (pAD), are designed for the identification and confirmation of protein interactions. Bait and prey proteins are expressed as GAL4 fusions with c-Myc and hemagglutinin epitope tags in these vectors, respectively. We first fused *gtf1* and *gtf2* with the DNA BD of pBD and DNA AD of pAD, respectively, using primer pairs listed in Table 3. The plasmid set pAD-*gtf1* and pBD-*gtf2* or plasmid set pAD-*gtf2* and pBD-*gtf1* was cotransformed into yeast strain AH109. Cotransformed yeast cells were streaked on a Leu/Trp/His/Ade amino acid-deficient SD agar plate to score protein-protein interactions.

In vitro GST pull-down assays. The in vitro GST pull-down assay can determine direct protein-protein interaction in vitro. We first constructed GST-Gtf1 and GST-Gtf2 fusion plasmids. pBD-*gtf1* or -*gtf2* was digested with BamHI and XhoI to release *gtf1* and *gtf2* ORFs, which were subcloned into pGEX5p1 vector (Amersham) to generate Gtf1- and Gtf2-GST in-frame fusion proteins, respectively. The GST fusion proteins were purified using glutathione Sepharose 4B beads (Amersham) following the manufacturer's instructions. In brief, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG)-induced cell pellets were lysed in the presence of 1 mM dithiothreitol and 1 mg/ml lysozyme by sonication. The cell lysates free of cell debris were mixed with 200 μl NETN buffer (20 mM Tris-HCl [pH 7.2], 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40, and protease inhibitor cocktail [1:20, vol/vol]) equilibrated GST beads. The mixture was incubated at 4°C overnight. The beads bound with GST or GST fusion proteins were washed with NETN buffer five times. Concentrations of GST fusion proteins were analyzed by SDS-PAGE followed by Coomassie blue staining.

The c-Myc-tagged in vitro-translated protein products of the full-length *gtf1* and *gtf2* genes were generated using TnT Quick Coupled transcription/translation system (Promega). One microgram of supercoiled DNA from pGBK7, pGBK7-Gtf1, and pGBK7-Gtf2 was used in the reaction mixtures that have 40 mM methionine. Approximately 5 μg of GST or GST fusion proteins in NETN washing buffer was mixed with 5 μl of in vitro-translated c-Myc fusion proteins in a final volume of 200 μl NETN binding buffer and incubated on a rotary shaker at 4°C overnight. The beads were washed three times with 600 μl NETN washing buffer, and the bound proteins were eluted by boiling in the SDS sample loading buffer and subjected to Western blotting analysis with anti-c-Myc antibody.

Construction of transposon insertion mutants in the *gtf1* fragment in *E. coli*. Plasmid *gtf1-2*-pGEX6p1 was used as a template to generate the construct. A T7 tag was fused into 3′ end of *gtf2* for the planned interaction study. There is an internal restriction enzyme site, BglII, near the 3′ end of *gtf2*. Using the primer pair BglII-*gtf2*-FF and XhoI-*gtf2*-T7-RR, a partial *gtf2* fragment with a T7 tag was amplified from pAL200. The amplified fragment was digested with BglII and XhoI and then ligated into pAL200 to generate pAL205, which has a T7 tag fused at the 3′ end of *gtf2*. A similar strategy was used to generate a negative control plasmid, pAL206, with pAL201 as a template.

Construction of Tn*5* transposon insertion mutants in the *gtf1* fragment in *E. coli* was carried out using the EZ-Tn*5* in-frame linker insertion kit (Epicentre). The kit is designed to rapidly produce random 57-bp (19 amino acids) insertions into a cloned DNA. Since the inserted 19 codons are designed to be readable in all three reading frames, the transposition generates nonpolar mutants.

As a NotI restriction enzyme digestion step is required to remove the kanamycin-resistant cassette from the insertional mutant constructs after transposition and there is a NotI site in the pGEX6p1 plasmid sequence, we first blunted the NotI site of the template plasmid pAL205 (*gtf1-2*-T7-pGEX6p1) by ligation of NotI-digested and T4 DNA polymerase-treated pAL205. The modified pAL205 was used to construct the transposon insertion mutants. The transposon mutants with Tn*5*-Km insertions were selected by their ability to resist to both ampicillin and kanamycin antibiotics. The insertion sites were identified by col-

TABLE 4. Characterization of Tn*5* transposon insertion mutants in the *gtf1* gene

Mutant	Insertion position (aa)[a]	MAb E2 mobility shift	sWGA (GlcNAc modification)	Interaction(s)
AL406/pAL205	NA	+	+	++
AL407/pAL206	NA	−	−	−
AL408/pAL207	31	−	−	−
AL409/pAL208	65	−	−	+
AL410/pAL209	159	+	+	++
AL411/pAL300	416	−	−	++

[a] NA, not applicable.

ony PCR using transposon- and Gtf1-specific primers NotI/KAN-3 RP-2 and pGEX6p1*gtf1*-F(910) (Table 3). The kanamycin-resistant cassette was removed by NotI digestion of plasmids prepared from selected transposon mutants. The exact transposition site was determined by sequencing. Four transposon insertion plasmids, pAL207, pAL208, pAL209, and pAL300 (Table 4), were chosen and used for cotransformation with pAL81 to generate AL408, -409, -410, and -411.

LC/MS/MS spectrometric analyses. Tandem-MS (MS/MS) analyses were performed with a Q-Tof2 mass spectrometer (Micromass, Manchester, United Kingdom) using electrospray ionization. Excised proteins that had undergone a 16-h tryptic digestion at 37°C were purified using ZipTips to concentrate and desalt the samples. The samples were then analyzed by liquid chromatography-MS/MS (LC/MS/MS). LC was performed using an LC Packings Ultimate LC Switchos microcolumn switching unit and Famos autosampler (LC Packings, San Francisco, CA). The samples were concentrated on a 300-μm-inside diameter C_{18} precolumn at a flow rate of 10 μl/min with 0.1% formic acid and then flushed onto a 75-μm-inside diameter C_{18} column at 200 μl/min with a gradient of 5 to 100% acetonitrile (0.1% formic acid) in 30 min. The nano-1c interface was used to transfer the 1c eluent into the mass spectrometer. The Q-Tof2 mass spectrometer was operated in the automatic switching mode, whereby multiply charged ions were subjected to MS/MS if their intensities rose above 6 counts. The tandem mass spectra were processed with the MassLynx MaxEnt 3 software.

RESULTS

Gtf1 and Gtf2 mutants exhibit a similar phenotype. Gtf1 is required for Fap1 glycosylation (34). Gtf2 shares some homology with other glycosyltransferases; however, its function in Fap1 glycosylation and its relationship with Gtf1 are not known. We inactivated the *gtf2* gene and also constructed a double mutant of the *gtf1* and *gtf2* genes in *S. parasanguinis* and compared these two mutants with the previously characterized *gtf1* mutant (34). As Fap1 and Fap1 precursors (32) have the same subcellular distribution pattern in *S. parasanguinis* (34), culture supernatant fractions were used to determine Fap1 expression. Wild-type *S. parasanguinis* FW213 expressed a mature 200-kDa Fap1 (Fig. 1A, lane 1). The *gtf2* mutant and the double mutant (*gtf1 gtf2*) behaved like the *gtf1* mutant and failed to express any mature 200-kDa protein. Instead, all of these mutants generated a high-molecular-mass Fap1 protein (360 kDa) that was detected by a peptide-specific MAb, E42 (Fig. 1A, lanes 3, 7, and 11). This high-molecular-mass Fap1 did not react with either of the glycan-specific MAbs F51 (Fig. 1B, lanes 3, 7, and 11) and D10 (data not shown), suggesting it has defects in Fap1 glycosylation.

Furthermore, concurrent inactivation of both *gtf1* and *gtf2* did not result in a phenotype (Fig. 1A and B, lanes 11) that was different from that produced by the single-insertion mutants (Fig. 1A and B, lanes 3 and 7). Introduction of plasmid-borne *gtf1*, *gtf2*, or a *gtf1 gtf2* fragment into their corresponding mutants restored the expression of the 200-kDa mature Fap1 protein (Fig. 1A and B, lanes 6, 10, and 14). Introduction of an empty plasmid, GFP-pVPT, or plasmids harboring the other gene (*gtf2*-pVPT into the *gtf1* mutant or *gtf1*-pVPT into the *gtf2* mutant) failed to restore Fap1 glycosylation (Fig. 1A and B, lanes 4, 5, 8, 9, 12, and 13). These results demonstrated that both Gtf1 and Gtf2 are required for Fap1 glycosylation and they cannot cross-complement each other, suggesting the two putative glycosyltransferases function in concert.

To confirm the high-molecular-mass Fap1 was not glycosylated, wild-type FW213, and *gtf1* and *gtf2* mutants were probed with lectin sWGA and a general carbohydrate stain. Only mature 200-kDa Fap1 in wild-type FW213 reacted with sWGA as well as the general carbohydrate stain (Fig. 1C and D, lane 2). The high-molecular-mass Fap1 species in the *gtf1* and *gtf2* mutants did not react with either reagent (Fig. 1C and D, lanes 3 and 4). In contrast, it reacted with the peptide-specific MAb E42 as a 360-kDa Fap1 precursor (Fig. 1E, lanes 3 and 4), suggesting that the high-molecular-mass Fap1 was not glycosylated.

The recombinant Fap1ΔRII is modified with GlcNAc-containing oligosaccharide(s). Fap1 is encoded by an 8-kb ORF and has two serine-rich repeat regions. These two repeat regions differ in size; however, they are identical in their amino acid compositions (35). It is difficult to work with full-length

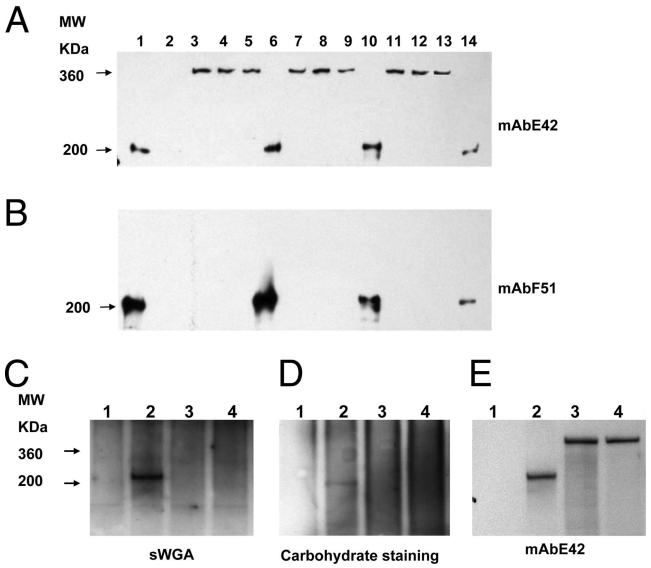

FIG. 1. Western blot analyses of Fap1 expression by *gtf1*, *gtf2*, and *gtf1 gtf2* mutants and their derivatives. Culture supernatants were prepared from wild-type FW213 (lane 1), VT1393 (*fap1* mutant; lane 2), VT1583 (*gtf1* mutant; lane 3), AL51 (*gtf1* mutant, pVPT; lane 4), AL52 (*gtf1* mutant, pVPT-*gtf2*; lane 5), AL50 (*gtf1* mutant, pVPT-*gtf1*; lane 6), AL63 (*gtf2* mutant; lane 7), AL64 (*gtf2* mutant, pVPT; lane 8), AL65 (*gtf2* mutant, pVPT-*gtf1*; lane 9), AL66 (*gtf2* mutant, pVPT-*gtf2*; lane 10), AL74 (*gtf1 gtf2* mutant; lane 11), AL75 (*gtf1 gtf2* mutant, pVPT-*gtf1*; lane 12), AL76 (*gtf1 gtf2* mutant, pVPT-*gtf2*; lane 13), and AL77 (*gtf1 gtf2* mutant, pVPT-*gtf1* and -*2*; lane 14); separated by electrophoresis through 4 to 12% polyacrylamide gradient gels; and subjected to Western blot analyses with peptide-specific MAb E42 (A) and glycan-specific MAb F51 (B). The culture supernatants prepared from VT1393 (*fap1* mutant; lane 1), wild-type FW213 (lane 2), VT1583 (*gtf1* mutant; lane 3), and AL63 (*gtf2* mutant; lane 4) were probed with lectin sWGA (C), carbohydrate staining (D), and MAb E42 (D), respectively. MW, molecular mass.

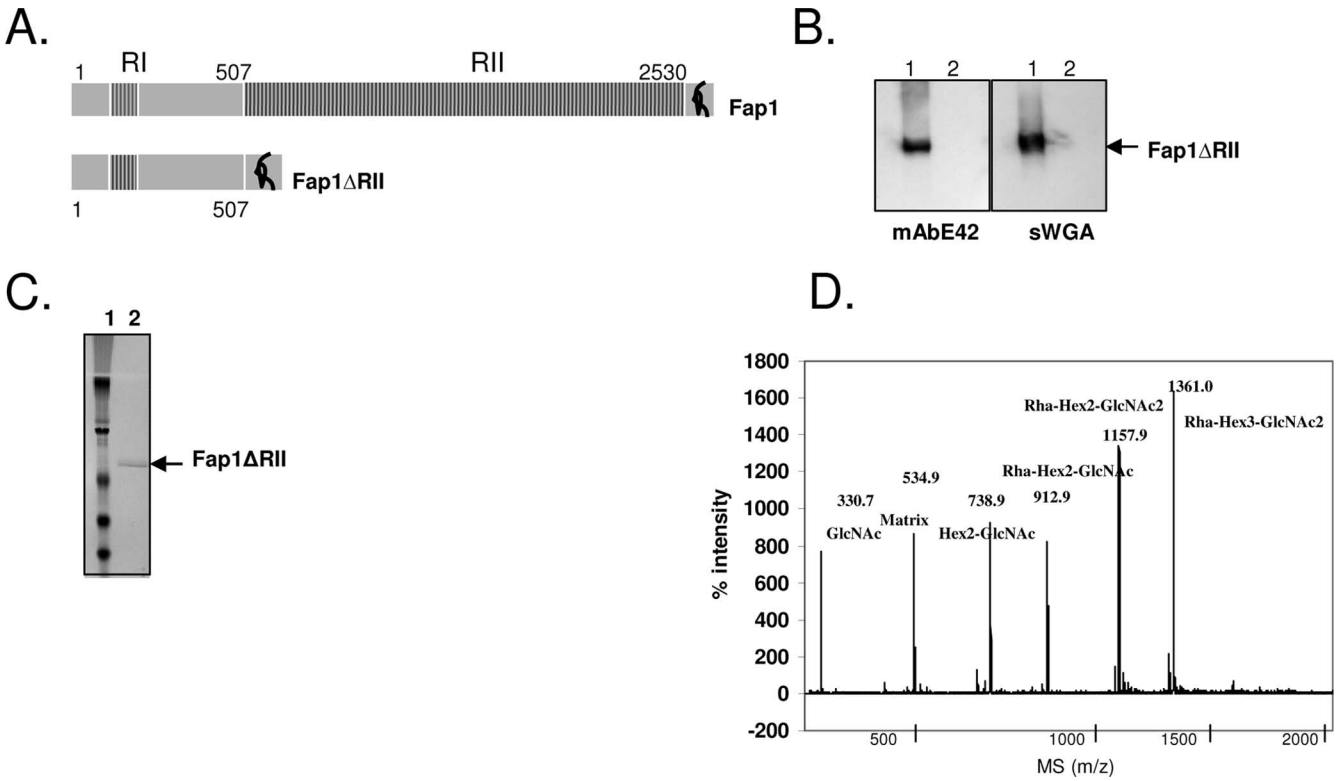

FIG. 2. Characterization of the recombinant Fap1 (Fap1ΔRII) that contains only repeat region I. (A) Diagram of Fap1 and Fap1ΔRII constructs. The *fap1*ΔRII construct was generated by deletion of the entire RII region from the intact *fap1*. (B) Fap1ΔRII is glycosylated by GlcNAc-containing sugar residues. The *fap1*ΔRII plasmid pAL81 was transformed into the *fap1*-null mutant VT1393 to generate a recombinant strain, AL81. AL81 (lane 1) and VT1393 (lane 2) were analyzed by Western blotting with MAb E42 and lectin sWGA (B). (C) Purification of Fap1ΔRII. Fap1ΔRII was purified by MAb E42 affinity chromatography from culture supernatants of AL81 and analyzed by Coomassie blue staining. (D) Glycosyl linkage analysis of Fap1ΔRII. The purified Fap1ΔRII protein was subjected to β-elimination, and the released oligosaccharides were analyzed by MALDI-TOF MS.

Fap1. Therefore, we engineered and expressed a new recombinant plasmid, pAL80, in which the repeat region II was deleted (Fig. 2A). The truncated Fap1, designated Fap1ΔRII, was expressed in recombinant strain AL81 and detected by MAb E42 and lectin sWGA (Fig. 2B, lanes 1). As sWGA is specific for GlcNAc, these data suggest Fap1ΔRII is glycosylated by GlcNAc-containing oligosaccharides. Fap1ΔRII was purified to near homogeneity by affinity chromatography (Fig. 2C, lane 2). Monosaccharide composition analyses of this protein revealed the presence of GlcNAc, glucose, galactose, and rhamnose at a ratio of 60:21:4:6. These sugar residues are also found in mature Fap1 (27), and the ratio is similar to that of full-length Fap1, indicating Fap1ΔRII was a useful model to study Fap1 glycosylation. Glycosyl linkage analyses of the purified Fap1ΔRII (Fig. 2D) revealed that the recombinant Fap1 was modified by mixed oligosaccharides and all the oligosaccharides started with GlcNAc, suggesting that the initial sugar residues attached to the peptide contain GlcNAc.

Both Gtf1 and Gtf2 were essential for Fap1ΔRII glycosylation in a functional *E. coli* glycosylation system. The above experiments indicated that Fap1ΔRII was glycosylated with GlcNAc-containing oligosaccharides. Gtf1 and Gtf2 were essential for Fap1 glycosylation in *S. parasanguinis*. Therefore, we hypothesized Gtf1 and Gtf2 were the glycosyltransferases to transfer the GlcNAc-containing oligosaccharides to the recombinant Fap1ΔRII. To test this hypothesis, we developed an in vivo glycosylation system in *E. coli* using Fap1ΔRII as the substrate and Gtf1 and Gtf2 as enzymes. Coexpression of the two plasmids in *E. coli* led to the production of a Fap1ΔRII doublet that migrated at the 75- and 83-kDa positions when probed with MAb E42 (Fig. 3A, lane 4). The GlcNAc-specific lectin sWGA reacted with the two same protein bands (Fig. 3B, lane 4). However, the bands did not react with any other lectins tested, including concanavalin A, *Dolichos biflorus* agglutinin, peanut agglutinin, *Ricinus communis* agglutinin I, soybean agglutinin, and *Ulex europaeus* agglutinin I. As these lectins are specific for glucose, mannose, galactose, *N*-acetylgalac-

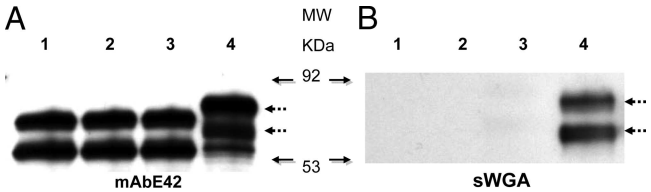

FIG. 3. Analyses of Fap1ΔRII expression in an *E. coli* glycosylation system. *E. coli* strains carrying pAL81 were cotransformed with plasmids pGEX6p1 (lane 1), pAL201 (lane 2), pAL202 (lane 3), and pAL200 (lane 4) and subjected to immunoblot analysis with MAb E42 (A) and GlcNAc-reactive lectin sWGA (B). MW, molecular mass.

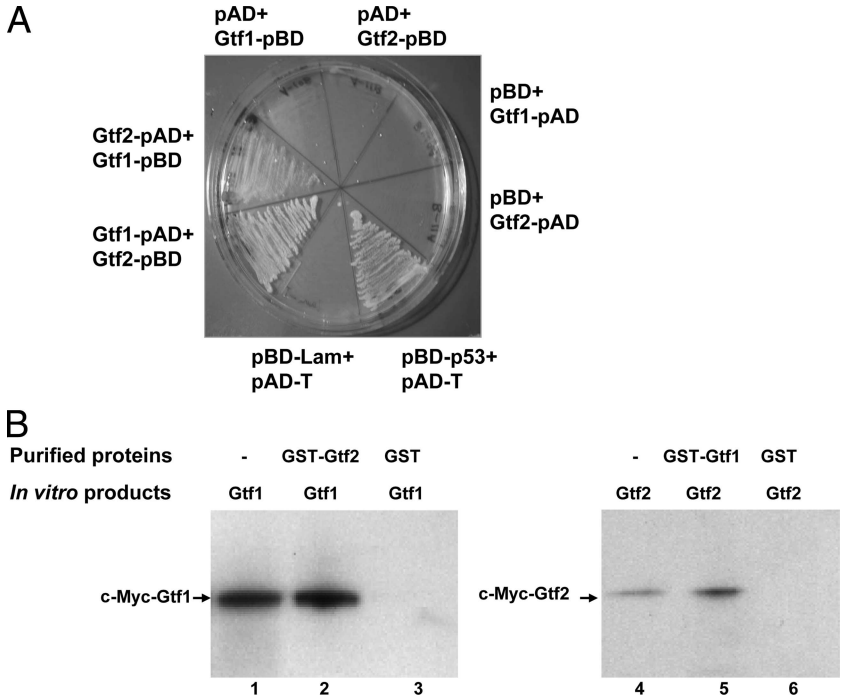

FIG. 4. Interaction between Gtf1 and Gtf2 determined by yeast two-hybrid and in vitro GST pull-down assays. (A) Yeast two-hybrid analysis of the interaction between Gtf1 and Gtf2. The full-length *gtf1* and *gtf2* genes were cloned into yeast two-hybrid reporter vectors pBD and pAD to construct pAD-*gtf1*, pBD-*gtf1*, pAD-*gtf2*, and pBD-*gtf2*, respectively. AH109 yeast was transformed with different combinations of bait and prey plasmids. The resulting growth of cotransformants was used to score protein-protein interactions. (B) In vitro GST pull-down assays for interactions between purified GST fusion proteins and in vitro-translated Gtf1 or Gtf2 products. The purified GST, GST-Gtf2, or GST-Gtf1 glutathione Sepharose beads were incubated with in vitro-translated c-Myc-Gtf1 (A) and c-Myc-Gtf2 (B) to pull down the interacting partners, and the captured protein complexes were subjected to Western blot analyses with c-Myc antibody. Inputs represented in vitro-translated c-Myc-Gtf1 (A) and c-Myc-Gtf2 (B) and serve as positive controls.

tosamine, and fucose, respectively, and did not react with Fap1ΔRII, we concluded Fap1ΔRII was only glycosylated by GlcNAc-containing oligosaccharides in *E. coli.* Deletion of either one or both Gtfs mitigated the Fap1 glycosylation, as determined by sWGA blotting (Fig. 3B, lanes 1 to 3), and the protein bands recognized by MAb E42 migrated to lower-molecular-mass positions (Fig. 3A, lanes 1 to 3). These results demonstrated that both Gtf1 and Gtf2 were required for Fap1ΔRII glycosylation with GlcNAc-containing oligosaccharides in the *E. coli* glycosylation system, supporting our early conclusion that both Gtf1 and Gtf2 were required for Fap1 glycosylation in *S. parasanguinis*.

Interaction between Gtf1 and Gtf2 revealed by a yeast two-hybrid analysis. As *gtf1* and *gtf2* mutants have very similar phenotypes and *gtf1* and *gtf2* cannot cross-complement each other, it is likely that they are interdependent in the Fap1 biosynthetic pathway. We hypothesized that Gtf1 and Gtf2 formed an enzyme complex to modulate Fap1 glycosylation. To test this, we used the yeast two-hybrid analysis to detect protein-protein interactions between Gtf1 and Gtf2. Numerous colonies were observed when the plasmid pair pAD-*gtf1* and pBD-*gtf2* or pAD-*gtf2* and pBD-*gtf1* were cotransformed. Their growth was comparable to that of the positive control (pBD-p53+ and pAD-T), suggesting Gtf1 interacts with Gtf2. The cotransformants of pAD-*gtf1* and pBD, pBD-*gtf1* and pAD, pAD-*gtf2* and pBD, and pBD-*gtf2* and pAD, along with the negative control (pBD-lam+ and pAD-T), failed to grow on the selective media (Fig. 4A), indicating that there was no self-activation of Gtf1 or Gtf2. Thus, we concluded that Gtf1 and Gtf2 specifically interacted with each other.

Direct interaction between Gtf1 and Gtf2 determined by in vitro GST pull-down assays. Yeast two-hybrid analysis demonstrated that Gtf1 interacts with Gtf2. To determine the direct interaction between Gtf1 and Gtf2, we performed in vitro GST pull-down assays. Gtf1 and Gtf2 were expressed as GST fusion proteins, and the purified fusion proteins were used to capture the in vitro-translated Gtf2- and Gtf1-c-Myc fusion proteins. GST-Gtf2 interacted strongly with c-Myc-Gtf1 (Fig. 4B, lane 2) and vice versa (Fig. 4B, lane 5), whereas GST did not interact with either of the c-Myc fusion proteins (lanes 3 and 6), demonstrating Gtf1 and Gtf2 directly interacted with each other in vitro.

Interactions between Gtf1 and Gtf2 in vivo in a functional Fap1ΔRII glycosylation system. Gtf1 and Gtf2 were required for Fap1ΔRII glycosylation in *E. coli*. We identified the interaction between Gtf1 and Gtf2 by yeast two-hybrid and GST pull-down analyses. To determine the interaction between Gtf1 and Gtf2 in vivo where the glycosylation occurs, we characterized the functional Fap1 glycosylation system that harbors Fap1ΔRII substrate (pAL81) and Gtf1-Gtf2 derivatives (pAL200, pAL201, or pAL202). In the presence of both Gtf1 and Gtf2, two proteins were copurified (Fig. 5A, lane 4): the top one migrated at an 86-kDa position corresponding to the predicted GST-Gtf1 fusion protein, and the bottom one mi-

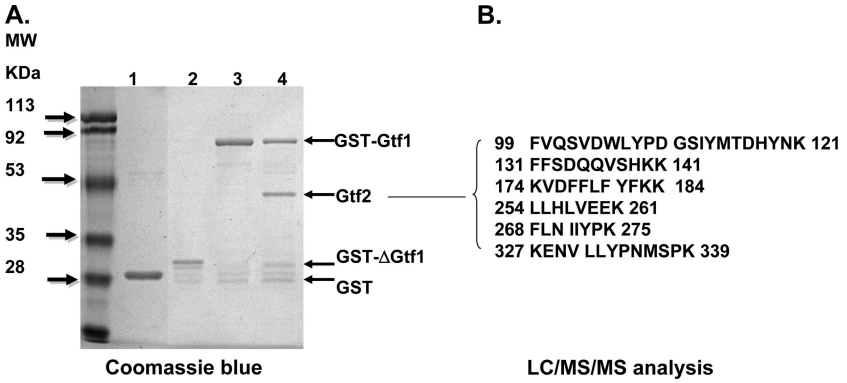

FIG. 5. Gtf1 and Gtf2 interact with each other in the *E. coli* glycosylation system. (A) In vivo GST pull down. The recombinant Fap1 substrate plasmid pAL81 was cotransformed with various pGEX6p1 derivatives into *E. coli* to generate a series of recombinant strains, AL400/pGEX6p1 (lane 1), AL402/pGEX6p1-*gtf1*(Δ21–485)-*2* (lane 2), AL403/pGEX6p1-*gtf1-2*(Δ15–417) (lane 3), and AL401/pGEX6p1-*gtf1-2* (lane 4). GST fusion proteins were purified from these recombinant strains and stained with Coomassie blue. MW, molecular mass. (B) MS analysis of Gtf2. A 50-kDa protein was copurified with GST-Gtf1, excised, and subjected to LC/MS/MS (B). Six tryptic peptides identified by MS matched with predicted peptides from Gtf2. Numbers indicate the amino acid positions in Gtf2.

grated with a size of 50 kDa corresponding to the predicted Gtf2 protein. This 50-kDa protein band was absent when the *gtf2* gene was deleted (Fig. 5A, lane 3). LC/MS/MS mass spectrometry analysis of this excised 50-kDa band identified this protein as Gtf2 (Fig. 5B). This 50-kDa band could not be copurified when we attempted to purify GST (Fig. 5A, lane 1) or a truncated GST-Gtf1 fusion protein (Fig. 5A, lane 2). These results suggested Gtf1 interacted with Gtf2 specifically in the in vivo Fap1ΔRII glycosylation system.

The N-terminal region of Gtf1 is important for the interaction and required for the glycosylation of Fap1ΔRII. Gtf1 interacted with Gtf2. In order to map the Gtf1 domain responsible for the interaction in detail, we randomly inserted 19 aa in Gtf1 using an in-frame transposon EZ-Tn*5* insertion system. Four different mutants with the transposon elements inserted in the *gtf1* region of pAL205 were identified and used to examine the effect of insertions on the interaction between Gtf1 and Gtf2. The plasmids harboring a nonpolar insertion at

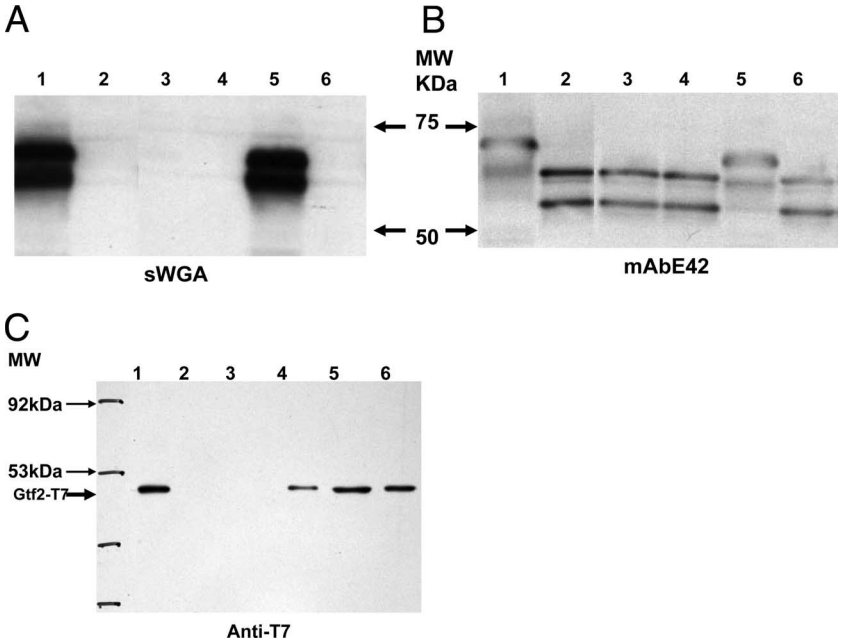

FIG. 6. The Gtf1 N-terminal region is critical for Gtf1 and Gtf2 interaction and Fap1 glycosylation. A variety of plasmids generated by using the EZ-Tn*5* transposon system (Table 4) were cotransformed with pAL81 into *E. coli* to construct a positive control strain, AL406/pGEX6p1-*gtf1-2*T7 (lane 1); a negative control, AL407/pGEX6p1-*gtf2*T7 (lane 2); and several in-frame insertional mutants, AL408 *gtf1*::Tn*31-gtf2* (lane 3), AL409 *gtf1*::Tn*65-gtf2* (lane 4), AL410 *gtf1*::Tn*159-gtf2* (lane 5), and AL411 *gtf1*::Tn*416-gtf2* (lane 6). Cotransformants were subjected to immune blotting with lectin sWGA (A) and MAb E42 (B). GST fusion proteins were purified from those cotransformants and analyzed by Western blotting assays with anti-T7 antibody (C). MW, molecular mass.

amino acid residues 31, 65, 159, and 416 of Gtf1 were designated as pAL207, pAL208, pAL209, and pAL300 (Table 4), respectively. These plasmids were cotransformed with the Fap1ΔRII substrate plasmid pAL81 to generate corresponding mutants AL408, AL409, AL410, and AL411 (Table 4). The expression and glycosylation profile of Fap1ΔRII of these mutants was determined by MAb E42 and lectin sWGA, respectively. Two mutants (AL408 and AL409) that have the insertions at the N-terminal region of Gtf1 failed to glycosylate Fap1ΔRII with GlcNAc-containing oligosaccharides (Fig. 6A, lanes 3 and 4). This phenotype was similar to that of the mutant AL411 (Fig. 6A, lane 6), which has the insertion at the C-terminal region of Gtf1. The Fap1ΔRII of these three mutants migrated at the same position as that of the negative control (Fig. 6B, lanes 3, 4, 6, and 2). Another mutant, AL410, which has the insertion at the amino acid residue 159 position, did not alter the modification pattern compared with the positive control AL406 (Fig. 6A and B, lanes 5 and 1), indicating the insertion in this position had no effect on Fap1ΔRII glycosylation.

A sequence similarity search revealed the C-terminal region of Gtf1 has a highly conserved glycosyltransferase catalytic domain; this may explain why insertion in this region (position 416) impaired Fap1 glycosylation. However, the function of the N-terminal domain of Gtf1 is not clear. The interaction between Gtf1 and Gtf2 is required for Fap1 glycosylation. Therefore, we hypothesized the N-terminal region of Gtf1 modulated its interaction with Gtf2. To test this, we performed in vivo pull-down experiments in the Fap1ΔRII glycosylation system. GST-Gtf1 fusion proteins were purified and adjusted to the same concentration semiquantitatively based on Coomassie blue staining of SDS-PAGE gel. We analyzed the copurified Gtf2 by Western blotting using T7 antibody and found the Gtf2-T7 fusion protein was absent from or dramatically reduced in AL408 and AL409 (Fig. 6C, lanes 3 and 4), suggesting that the interaction between Gtf1 and Gtf2 was abrogated in AL408 and significantly reduced in AL409. Notably, the interaction between Gtf1 and Gtf2 in AL408 was comparable to that in the negative control (Fig. 6C, lane 2), indicating the interaction was crucial for Fap1 glycosylation. Meanwhile, a putative catalytic mutant, AL411, failed to glycosylate Fap1ΔRII (Fig. 6B, lane 6), but the mutation did not alter the protein-protein interaction (Fig. 6C, lane 6). These data suggested that amino acid position 416 was critical for the Gtf1 enzyme activity but not required for the protein-protein interaction.

In conclusion, we identified two Gtf1 regions that were important for Fap1 glycosylation: the N-terminal region was critical for the enzyme complex formation, whereas the C-terminal domain was needed for the catalytic function of Gtf1.

DISCUSSION

In this study, we determined (i) GlcNAc was among the first sugar residues transferred to the Fap1 peptide and (ii) the formation of a protein complex by two putative glycosyltransferases was required for the transfer. We engineered a recombinant Fap1 molecule that contains the serine-rich repeat region (RI) of Fap1 to study the glycosylation of Fap1. This recombinant molecule displayed a monosaccharide composition profile similar to mature Fap1. The predominant monosaccharide residues associated with the recombinant Fap1, *N*-acetylglucosamine and glucose, are also found in mature Fap1. Glycosyl linkage analyses suggested that all of the oligosaccharides were initiated from a GlcNAc residue, indicating that GlcNAc is the initial sugar transferred to the Fap1 peptide backbone.

To test the hypothesis that Gtf1 and Gtf2 are initial glycosyltransferases responsible for the transfer of GlcNAc-containing oligosaccharide(s), we established an in vivo Fap1 glycosylation system in *E. coli*. This system utilized the recombinant Fap1 (Fap1ΔRII) as a substrate and Gtf1 and Gtf2 as the only streptococcus-derived putative glycosyltransferases. This model excludes the effect of other streptococcal glycosyltransferases on Fap1 glycosylation and has allowed us to dissect the initial glycosylation step. Using this system, we confirmed that putative glycosyltransferases Gtf1 and Gtf2 are essential for production of the GlcNAc-containing oligosaccharides, supporting the notion that GlcNAc is among the first sugar molecules transferred to the peptide backbone. Interestingly, the oligosaccharides released from the recombinant Fap1 isolated from *S. parasanguinis* were not homogenous. The question arises as to why the recombinant Fap1 modification is homogenous in *E. coli* and heterogeneous in *S. parasanguinis*. A plausible explanation is that other putative glycosyltransferases identified from the Fap1 locus that are important for the subsequent Fap1 glycosylation in *S. parasanguinis* are missing in this recombinant *E. coli* strain. The GlcNAc residue-containing glycan moiety has also been identified in other serine-rich proteins, including GspB of *S. gordonii* (1) and SrpA of *S. sanguinis* (21). Therefore, it is possible that the transfer of GlcNAc-containing sugar residues to the serine-rich regions by Gtf1 and Gtf2 homologues may represent a common theme as the Gtf1 and Gtf2 molecules are highly conserved throughout streptococci and staphylococci that express Fap1-like proteins. Furthermore, Gtf1 and Gtf2 isolated from *S. sanguinis* and *S. pneumoniae* were able to glycosylate Fap1ΔRII with GlcNAc-containing oligosaccharides (data not shown), supporting the concept that Gtf1 and -2 homologues are capable of the transfer.

Genetic analyses of Gtf1 and Gtf2 mutants in both *S. parasanguinis* and *E. coli* systems suggested the two putative glycosyltransferases were required for Fap1 glycosylation. This result is consistent with a previous report that Gtf1 and -2 homologues are important for GspB glycosylation in *S. gordonii* (31). Protein glycosylation is modulated by glycosyltransferase-mediated initiation and elongation in a stepwise manner. Many glycosyltransferases of mammalian and eukaryotic cells are functionally and physically associated with each other to facilitate the glycosylation process (16). We have shown Gtf1 and Gtf2 formed a protein complex by yeast two-hybrid and in vitro GST pull-down assays. Furthermore, the interaction between two putative glycosyltransferases appears to be essential in the in vivo glycosylation system that simulates the initial Fap1 glycosylation reaction. All of these results suggest the formation of Gtf1 and Gtf2 complex was required for the initial glycosylation. We have identified two Gtf1 domains that are differentially involved in their interaction with Gtf2 and Fap1 glycosylation. A Gtf1 N-terminal domain directly interacted with Gtf2, thereby modulating Fap1 glycosylation. The

C-terminal amino acid position 416 was not critical for this interaction; however, it was required for Fap1 glycosylation, suggesting it has a glycosyltransferase catalytic domain. Indeed, a domain search revealed the C-terminal region has a highly conserved glycosyltransferase activity motif.

GtfA and GtfB of *S. gordonii*, homologues of putative glycosyltransferases of *S. parasanguinis*, are critical for glycosylation of GspB (31). However, whether they interact with each other is not known. Given the amino acid sequence similarity observed among glycosyltransferases from a variety of streptococci and staphylococci, it is reasonable to assume the interaction between putative glycosyltransferases also exists in these streptococcal and staphylococcal species as well. It is not clear how the interaction between Gtf1 and Gtf2 mediates enzymatic activity that regulates Fap1 glycosylation. Conceivably, Gtf1 and Gtf2 are involved in sequential biosynthesis of Fap1 glycans and the interaction between Gtf1 and Gtf2 promotes the efficient sequential biosynthesis of Fap1 glycans. Alternatively, Gtf1 and Gtf2 may be an integral part of an enzyme complex. Perhaps Gtf2 functions like a chaperone and contributes a missing fold to Gtf1, making both essential for enzymatic activities. This hypothesis is in agreement with the fact that both *gtf1* and *gtf2* mutants showed very similar phenotypes: i.e., expression of a 360-kDa Fap1 precursor. Many proteins responsible for carbohydrate biosynthesis form heterocomplexes that facilitate glycan biogenesis. Biosynthesis of ganglioside precursors requires the assembly of distinct multiple enzyme complexes including glycosyltransferases (13). Two members of β1,3-*N*-acetylglucosaminyl-transferase family, β3Gn-T2 and -T8, interact with each other and can enhance corresponding enzymatic activities in vitro (24). However, whether the interaction between the two enzymes occurs in vivo has not been demonstrated. This is the first report that describes the importance of the interaction between two putative glycosyltransferases in the glycosylation of a bacterial adhesin. As many examples of protein-protein interaction between glycosyltransferases have been documented in eukaryotic systems, our findings indicate the formation of protein complexes by interactions between glycosyltransferases is a common theme in protein glycosylation, whether they are prokaryotic or eukaryotic in origin.

ACKNOWLEDGMENTS

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REFERENCES

1. **Bensing, B. A., B. W. Gibson, and P. M. Sullam.** 2004. The *Streptococcus gordonii* platelet binding protein GspB undergoes glycosylation independently of export. J. Bacteriol. **186:**638–645.
2. **Carlsson, J., H. Grahnen, G. Jonsson, and S. Wikner.** 1970. Establishment of *Streptococcus sanguis* in the mouths of infants. Arch. Oral Biol. **15:**1143–1148.
3. **Chen, Q., H. Wu, and P. M. Fives-Taylor.** 2004. Investigating the role of *secA2* in secretion and glycosylation of a fimbrial adhesin in *Streptococcus parasanguis* FW213. Mol. Microbiol. **53:**843–856.
4. **Chen, Q., H. Wu, R. Kumar, Z. Peng, and P. M. Fives-Taylor.** 2006. SecA2 is distinct from SecA in immunogenic specificity, subcellular distribution and requirement for membrane anchoring in *Streptococcus parasanguis*. FEMS Microbiol. Lett. **264:**174–181.
5. **Cole, R. M., G. B. Calandra, E. Huff, and K. M. Nugent.** 1976. Attributes of potential utility in differentiating among "group H" streptococci or *Streptococcus sanguis*. J. Dent. Res. **55:**A142–A153.
6. **de Graffenried, C. L., and C. R. Bertozzi.** 2004. The roles of enzyme localisation and complex formation in glycan assembly within the Golgi apparatus. Curr. Opin. Cell Biol. **16:**356–363.
7. **Dell, A.** 1990. Preparation and desorption mass spectrometry of permethyl and peracetyl derivatives of oligosaccharides. Methods Enzymol. **193:**647–660.
8. **Erickson, P. R., and M. C. Herzberg.** 1993. Evidence for the covalent linkage of carbohydrate polymers to a glycoprotein from *Streptococcus sanguis*. J. Biol. Chem. **268:**23780–23783.
9. **Fachon-Kalweit, S., B. L. Elder, and P. Fives-Taylor.** 1985. Antibodies that bind to fimbriae block adhesion of *Streptococcus sanguis* to saliva-coated hydroxyapatite. Infect. Immun. **48:**617–624.
10. **Fenno, J. C., A. Shaikh, and P. Fives-Taylor.** 1993. Characterization of allelic replacement in *Streptococcus parasanguis*: transformation and homologous recombination in a 'nontransformable' streptococcus. Gene **130:**81–90.
11. **Fives-Taylor, P. M., and D. W. Thompson.** 1985. Surface properties of *Streptococcus sanguis* FW213 mutants nonadherent to saliva-coated hydroxyapatite. Infect. Immun. **47:**752–759.
12. **Gibbons, R. J., and J. van Houte.** 1975. Dental caries. Annu. Rev. Med. **26:**121–136.
13. **Giraudo, C. G., and H. J. Maccioni.** 2003. Ganglioside glycosyltransferases organize in distinct multienzyme complexes in CHO-K1 cells. J. Biol. Chem. **278:**40262–40271.
14. **Kremer, B. H. A., M. van der Kraan, P. J. Crowley, I. R. Hamilton, L. J. Brady, and A. S. Bleiweis.** 2001. Characterization of the *sat* operon in *Streptococcus mutans*: evidence for a role of Ffh in acid tolerance. J. Bacteriol. **183:**2543–2552.
15. **Lawrence, M. L., M. M. Banes, P. Azadi, and B. Y. Reeks.** 2003. The *Edwardsiella ictaluri* O polysaccharide biosynthesis gene cluster and the role of O polysaccharide in resistance to normal catfish serum and catfish neutrophils. Microbiology **149:**1409–1421.
16. **Lehle, L., S. Strahl, and W. Tanner.** 2006. Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases. Angew. Chem. Int. Ed. Engl. **45:**6802–6818.
17. **Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans, and D. B. Clewell.** 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. Gene **19:**345–353.
18. **Marsh, P. D.** 1995. The role of microbiology in models of dental caries. Adv. Dent. Res. **9:**244–269.
19. **Mintz, K. P., and P. M. Fives-Taylor.** 2000. Development of a *rec*A− strain of *Actinobacillus actinomycetemcomitans*. J. Dent. Res. **79:**338.
20. **Obert, C., J. Sublett, D. Kaushal, E. Hinojosa, T. Barton, E. I. Tuomanen, and C. J. Orihuela.** 2006. Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. Infect. Immun. **74:**4766–4777.
21. **Plummer, C., H. Wu, S. W. Kerrigan, G. Meade, D. Cox, and C. W. Ian Douglas.** 2005. A serine-rich glycoprotein of *Streptococcus sanguis* mediates adhesion to platelets via GPIb. Br. J. Haematol. **129:**101–109.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. **Seifert, K. N., E. E. Adderson, A. A. Whiting, J. F. Bohnsack, P. J. Crowley, and L. J. Brady.** 2006. A unique serine-rich repeat protein (Srr-2) and novel surface antigen (epsilon) associated with a virulent lineage of serotype III Streptococcus agalactiae. Microbiology **152:**1029–1040.
24. **Seko, A., and K. Yamashita.** 2005. Characterization of a novel galactose beta1,3-N-acetylglucosaminyltransferase (beta3Gn-T8): the complex formation of beta3Gn-T2 and beta3Gn-T8 enhances enzymatic activity. Glycobiology **15:**943–951.
25. **Siboo, I. R., H. F. Chambers, and P. M. Sullam.** 2005. Role of SraP, a serine-rich surface protein of *Staphylococcus aureus*, in binding to human platelets. Infect. Immun. **73:**2273–2280.
26. **Smith, L. M., J. Z. Sanders, R. J. Kaiser, P. Hughes, C. Dodd, C. R. Connell, C. Heiner, S. B. Kent, and L. E. Hood.** 1986. Fluorescence detection in automated DNA sequence analysis. Nature **321:**674–679.
27. **Stephenson, A. E., H. Wu, J. Novak, M. Tomana, K. Mintz, and P. Fives-Taylor.** 2002. The Fap1 fimbrial adhesin is a glycoprotein: antibodies specific for the glycan moiety block the adhesion of *Streptococcus parasanguis* in an *in vitro* tooth model. Mol. Microbiol. **43:**147–157.
28. **Szymanski, C. M., and B. W. Wren.** 2005. Protein glycosylation in bacterial mucosal pathogens. Nat. Rev. Microbiol. **3:**225–237.
29. **Takahashi, Y., K. Konishi, J. O. Cisar, and M. Yoshikawa.** 2002. Identification and characterization of *hsa*, the gene encoding the sialic acid-binding adhesin of *Streptococcus gordonii* DL1. Infect. Immun. **70:**1209–1218.
30. **Takahashi, Y., E. Takashima, K. Shimazu, H. Yagishita, T. Aoba, and K. Konishi.** 2006. Contribution of sialic acid-binding adhesin to pathogenesis of experimental endocarditis caused by *Streptococcus gordonii* DL1. Infect. Immun. **74:**740–743.
31. **Takamatsu, D., B. A. Bensing, and P. M. Sullam.** 2004. Four proteins encoded in the *gspB-secY2A2* operon of *Streptococcus gordonii* mediate the intracellular glycosylation of the platelet-binding protein GspB. J. Bacteriol. **186:**7100–7111.

32. **Tao, L., D. J. LeBlanc, and J. J. Ferretti.** 1992. Novel streptococcal-integration shuttle vectors for gene cloning and inactivation. Gene **120:**105–110.
33. **Wacker, M., M. F. Feldman, N. Callewaert, M. Kowarik, B. R. Clarke, N. L. Pohl, M. Hernandez, E. D. Vines, M. A. Valvano, C. Whitfield, and M. Aebi.** 2006. Substrate specificity of bacterial oligosaccharyltransferase suggests a common transfer mechanism for the bacterial and eukaryotic systems. Proc. Natl. Acad. Sci. USA **103:**7088–7093.
34. **Wu, H., S. Bu, P. Newell, Q. Chen, and P. Fives-Taylor.** 2007. Two gene determinants are differentially involved in the biogenesis of Fap1 precursors in *Streptococcus parasanguis*. J. Bacteriol. **189:**1390–1398.
35. **Wu, H., and P. M. Fives-Taylor.** 1999. Identification of dipeptide repeats and a cell wall sorting signal in the fimbriae-associated adhesin, Fap1, of *Streptococcus parasanguis*. Mol. Microbiol. **34:**1070–1081.
36. **Wu, H., and P. M. Fives-Taylor.** 2001. Molecular strategies for fimbrial expression and assembly. Crit. Rev. Oral Biol. Med. **12:**101–115.
37. **Wu, H., K. P. Mintz, M. Ladha, and P. M. Fives-Taylor.** 1998. Isolation and characterization of Fap1, a fimbriae-associated adhesin of *Streptococcus parasanguis* FW213. Mol. Microbiol. **28:**487–500.
38. **Wu, H., M. Zeng, and P. Fives-Taylor.** 2007. The glycan moieties and the N-terminal polypeptide backbone of a fimbria-associated adhesin, Fap1, play distinct roles in the biofilm development of *Streptococcus parasanguinis*. Infect. Immun. **75:**2181–2188.